



#15  
Drop  
23-03

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No: 38005-0147

*In re* patent application of:

Evi KOSTENIS et al. Confirmation No.: 5658

Serial No.: 09/842,316 Group Art Unit: 1646

Filed: April 26, 2001 Examiner: John D. Ulm

For: EDG8 RECEPTOR, ITS PREPARATION AND USE

RECEIVED  
JAN 23 2003  
TECH CENTER 1600/2900

CLAIM FOR CONVENTION PRIORITY

Commissioner for Patents  
Washington, D.C. 20231

Sir:

The benefit of the filing date of the following prior foreign application filed in the following foreign country is hereby requested, and the right of priority provided in 35 U.S.C. § 119 is hereby claimed. In support of this claim, filed herewith is a certified copy of said original foreign applications:

**European Patent Application No. 00108858.2, filed April 26, 2000; and  
European Patent Application No. 00116589.3, August 1, 2000.**

January 22, 2003

Respectfully submitted,

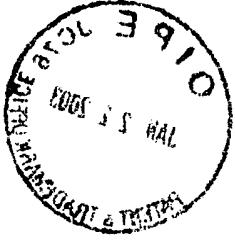
Patricia D. Granados  
Reg. No.: 33,683

HELLER EHRLMAN WHITE & McAULIFFE  
1666 K Street, NW, Suite 300  
Washington, DC 20006-1228  
(202) 912-2000 (telephone)



26633

PATENT TRADEMARK OFFICE



**THIS PAGE BLANK (USPTO)**



Europäisches  
Patentamt



Bescheinigung

European  
Patent Office

Certificate

Office européen  
des brevets

O.J.G.  
JAN 22 2003  
H.C.H.

Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

00108858.2

Der Präsident des Europäischen Patentamts;  
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets  
p.o.

Hatten

I.L.C. HATTEN-HECKMAN

DEN HAAG, DEN  
THE HAGUE, 15/11/00  
LA HAYE, LE



**THIS PAGE BLANK (USPTO)**



Europäisches  
Patentamt

European  
Patent Office

Office eur péen  
des brevets

**Blatt 2 d r Bescheinigung**  
**Sheet 2 of the certificate**  
**Page 2 de l'attestation**

Anmeldung Nr.:  
Application no.: **00108858.2**  
Demande n°:

Anmeldetag:  
Date of filing: **26/04/00**  
Date de dépôt:

Anmelder:  
Applicant(s):  
Demandeur(s):  
**Aventis Pharma Deutschland GmbH**  
**65929 Frankfurt am Main**  
**GERMANY**

Bezeichnung der Erfindung:  
Title of the invention:  
Titre de l'invention:  
**EDG8 receptor, its preparation and use**

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat: Tag: Aktenzeichen:  
State: Date: File no.  
Pays: Date: Numéro de dépôt:

Internationale Patentklassifikation:  
International Patent classification:  
Classification internationale des brevets:  
**C12N15/12, C07K14/705, C12Q1/68, G01N33/52, G01N33/50**

Am Anmeldetag benannte Vertragstaaten:  
Contracting states designated at date of filing: AT/BE/CH/CY/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE/TR  
Etats contractants désignés lors du dépôt:

Bemerkungen:  
Remarks:  
Remarques:

**THIS PAGE BLANK (USPTO)**

EPO - Munich  
41

26. April 2000

## EDG8 receptor, its preparation and use

The present invention relates to newly identified EDG8 receptors, polynucleotides encoding this receptor, polypeptides encoded by such polynucleotides the preparation and the use of thereof.

In an effort to identify new G-protein coupled receptors of the EDG (endothelial differentiation gene)-family a novel member of the EDG-family of G-protein coupled receptors, Human EDG8, was identified. The full-length clone was isolated and studies on chromosomal mapping, tissue expression and identification as a functional cellular receptor for sphingosine 1-phosphate were performed. Taken together, the data provide compelling evidence that EDG8 is the fifth receptor for sphingosine 1-phosphate, exclusively expressed in peripheral tissues, its presence in endothelial cells being responsible for the broad tissue distribution.

The lysolipid phosphate mediators lysophosphatidic acid (LPA) and sphingosin 1-phosphate (S1P) have attracted increasing attention as modulators of a variety of important biological functions (Moolenaar et al., 1997; Morris, 1999; Lynch, 1999) and their list of biological activities is continuously growing.

Among the biological responses to LPA is platelet aggregation (Durieux and Lynch, 1993; Moolenaar, 1994; Jalink et al., 1994; Siess et al., 1999; Gueguen et al., 1999), smooth muscle contraction, in vivo vasoactive effects, chemotaxis, expression of adhesion molecules on endothelial cells, increased tight junction permeability, activation of membrane ion channels and many others. The biochemical signalling events that mediate the cellular effects of LPA include stimulation of phospholipases, mobilization of intracellular  $\text{Ca}^{2+}$ , inhibition of adenylyl cyclase, activation of phosphatidylinositol 3-kinase, activation of the Ras-Raf-MAP kinase cascade and stimulation of Rho-GTPases.(Moolenaar et al., 1997)

S1P, in particular, is implicated in cell proliferation, induction/suppression of apoptosis, modulation of cell motility, angiogenesis, tumor invasiveness, platelet activation and neurite retraction. Cellular signalling by S1P involves activation of  $\text{PLC}\beta$  and subsequent intracellular  $\text{Ca}^{2+}$  release, activation of MAP-kinases,

activation of inward rectifying K<sup>+</sup>-channels and inhibition and/or activation of adenylyl cyclase.

Both, LPA and S1P are recognized to signal cells through a set of G-protein coupled receptors (GPCRs) known as EDG (endothelial differentiation gene)-receptors. The EDG-family of GPCRs currently comprises seven human members (EDG1-7) that fall into two major groups depending on their preference for the activating lipid-ligand: EDG1, 3, 5 and 6 preferentially interact with S1P, EDG2, 4 and 7 preferentially interact with LPA.

The assignment of specific biological functions to certain receptor subtypes is hampered by the fact that EDG receptors are expressed in an overlapping fashion, they activate multiple and in part identical signal transduction pathways, the selectivity for their activating ligands is not absolute, and medicinal chemistry is only poorly developed in that specific antagonists for dissecting the pharmacology of the individual subtypes are not available yet.

An important step to shed more light on the biological role of the individual receptor subtypes would be to identify the complete set of receptors that respond to the phospholipid mediators S1P and LPA.

The present invention relates to newly identified EGD8 receptors, polynucleotides encoding this receptor, polypeptides encoded by such polynucleotides the preparation and the use of thereof.

The present invention relates to an isolated polynucleotide comprising a nucleotide sequence that has at least 80 % identity to a nucleotide sequence encoding the polypeptide of SEQ ID NO. 2 or the corresponding fragment thereof; or a nucleotide sequence complementary to said nucleotide sequence.

Preferably, the polynucleotide is DNA or RNA.

Preferably, the nucleotide sequence of the polynucleotide is at least 80 % identical to that contained in SEQ ID NO. 1.

In another embodiment, the polynucleotide has the nucleotide sequence SEQ ID NO. 1. In another embodiment, the polynucleotide encodes the polypeptide of SEQ ID NO. 2 or a fragment thereof.

Another aspect to the invention relates to an expression system for the expression of EDG8. The EDG8 DNA or RNA molecule comprising an expression system wherein said expression system is capable of producing a polypeptide or a fragment thereof having at least 80 % identity with a nucleotide sequence encoding the polypeptide of SEQ ID NO. 2 or said fragment when said expression system is present in a compatible host cell.

The invention relates to a host cell comprising the expression system.

In another aspect, the invention relates to a process for producing a EDG8 polypeptide or fragment comprising culturing a host cell comprising the expression system under conditions sufficient for the production of said polypeptide or fragment. Preferably, the said polypeptide or fragment is expressed at the surface of said cell. The invention relates also to cells produced by this process.

The process preferably further includes recovering the polypeptide or fragment from the culture.

In another aspect, the invention relates to a process for producing a cell which produces a EDG8 polypeptide or a fragment thereof comprising transforming or transfecting a host cell with the expression system such that the host cell, under appropriate culture conditions, produces a EDG8 polypeptide or fragment.

In particular, the invention relates to an EDG8 polypeptide or a fragment thereof comprising an amino acid sequence which is at least 80 % identical to the amino acid sequence contained in SEQ ID NO. 2; in particular to an EDG8 polypeptide or a fragment thereof having amino acid sequence SEQ ID NO. 2 or a part thereof.

Further, the invention relates to a process for diagnosing a disease or a susceptibility to a disease related to expression or activity of EDG8 polypeptide comprising:

- a) determining the presence or absence of mutation in the nucleotide sequence encoding said EDG8 polypeptide in the genome of said subject; and/or
- b) analyzing for the presence or amount of the EDG8 polypeptide expression in a sample derived from said subject.

In addition, the invention relates to a method for identifying compounds which bind to EDG8 polypeptide comprising:

- a) contacting a cell comprising the expression system or a part of such a cell with a candidate compound; and
- b) assessing the ability of said candidate compound to bind to said cells.

Preferably, the method further includes determining whether the candidate compound effects a signal generated by activation of the EDG8 polypeptide at the surface of the cell, wherein a candidate compound which effects production of said signal is identified as an agonist.

In another embodiment of the invention, the method further includes determining whether the candidate compound effects a signal generated by activation of the EDG8 polypeptide at the surface of the cell, wherein a candidate compound which effects production of said signal is identified as an antagonist.

The invention also relates to an agonist or antagonist identified by such methods.

In another special embodiment of the method, the method further includes contacting said cell with a known agonist for said EDG8 polypeptide; and determining whether the signal generated by said agonist is diminished in the presence of said candidate compound, wherein a candidate compound which effects a diminution in said signal is identified as an antagonist for said EDG8 polypeptide. The known agonist is for example S1P, LPA and/or DHS1P. The invention also relates to an antagonist identified by the method.

The invention in addition, relates to a method of preparing a pharmaceutical composition comprising

- a) identifying a compound which is an agonist or an antagonist of EDG8,
- b) preparing the compound, and
- c) optionally mixing the compound with suitable additives.

The invention also relates to a pharmaceutical compound prepared by such a process.

In the study, we report about the cloning, chromosomal mapping, tissue expression and functional identification as a receptor for S1P of a novel GPCR, EDG8, the fifth functional receptor for sphingosine 1-phosphate.

In an effort to identify new G-protein coupled receptors of the EDG-family a database search with alignments of the currently known 18 members of this receptor family, comprising human EDG1-7 sequences up to the putative EDGs from Xenopus and Zebra-fish was performed. A multiple alignment of these sequences was created by CLUSTALW and used in a PSI-BLAST search to scan translated versions of human genomic DNA sequences, which were publicly available in the different EMBL sections. For translation of DNA into protein sequences, individual protein files within two respective STOP-codons were created and all proteins shorter than 50 amino acids were ignored. As the majority of GPCRs is unspliced searching for GPCRs within genomic sequences should bring about novel receptor proteins.

Performing a PSI-BLAST search, the various cDNAs and genomic contigs, respectively, for the human EDG1-7 receptors were identified, and an additional genomic hit with a high e-value, that was not identical to any of the published EDG-sequences. The nucleotide and amino acid sequence of the new putative GPCR are depicted in Fig.1A. Hydropathy analysis (hydrophobicity plot not shown) suggests a seven transmembrane protein with three alternating extra- and intracellular loops, assumed to be the heptahelix structure common to GPCRs.

To shed more light on the relationships involved in the molecular evolution of the EDG-receptor family, a grow tree phylogram was constructed using the neighbor joining method (GCG software) (Fig.1B) According to this phylogenetic tree, the human EDG-family can be divided into two distinct groups: EDG1, 3, 5 and 6 belonging to one, EDG2, 4 and 7 belonging to the other group. These two groups are discriminated further by their preference for different lipid ligands: EDG1, 3, 5, 6 are preferentially stimulated by sphingosin 1-phosphate (S1P), EDG2, 4 and 7 by lysophosphatidic acid (LPA). The newly identified sequence was named EDG8 for reasons of consistency with the existing human EDG-family nomenclature and

xhibited highest similarity (86.8%) to the rat nrg1-protein (Fig. 1B), a GPCR recently cloned by EST-expression profiling from a rat PC12 cell library (Glickman et al., 1999). The high similarity between EDG8 and the known sphingosin 1-phosphate (S1P) receptors EDG1, 3 and 5 (48-51%) (Fig. 1C) led us to test the hypothesis that EDG8 may be a functional S1P-receptor.

In testing for S1P receptor activity, the EDG8 cDNA was introduced into chinese hamster ovary (CHO) cells by transient transfection. CHO cells were chosen as they exhibit minimal responses to sphingosin 1-phosphate in concentrations up to 1  $\mu$ M but respond to S1P after transfection with the S1P preferring receptors EDG 1, 3 and 5 (Okamoto et al., 1998; Kon et al., 1999). To test functional receptor activity, it was decided to monitor the mobilization of intracellular Ca<sup>2+</sup> for three reasons:

- 1.) S1P has been reported to increase Ca<sup>2+</sup> in many cell types;
- 2.) the identification of EDG1, 3, 5 and 6 as receptors for S1P has provided the molecular basis for a GPCR mediated mechanism and the receptors are known to mediate intracellular Ca<sup>2+</sup> through either PTX-sensitive Gi proteins or the PTX-insensitive Gq/11 pathway;
- 3.) all currently known S1P-responding EDG-receptors (except EDG6) are present in endothelial cells (A. Niedernberg et al., submitted), in which intracellular Ca<sup>2+</sup> release is a major pathway in the generation of NO, an important factor in vascular biology.

Thus, identification of the complete set of S1P receptors, involved in intracellular Ca<sup>2+</sup> mobilization could help clarify the role of the individual subtypes in endothelial cell signalling.

Fig.2 depicts measurement of the intracellular Ca<sup>2+</sup> concentration, mediated by S1P via the different S1P-receptors EDG1, 3, 5, 6 and the putative S1P-receptor EDG8, cotransfected in CHO cells with empty vector DNA as a control or various G-protein  $\alpha$  subunits. Intracellular Ca<sup>2+</sup> concentrations were recorded as real time measurements using the Fluorescence plate imaging reader (FLIPR, Molecular Devices). As has been reported for EDG1, 3 and 5, S1P elicited intracellular Ca<sup>2+</sup> signals that did not require cotransfection of a G-protein  $\alpha$  subunit. As already known for a large number of Gq-coupled receptors, coexpression of Gq augments the EDG1 and 5-mediated Ca<sup>2+</sup>-release as compared with the Ca<sup>2+</sup> signal induced by

stimulation of endogenous G<sub>q</sub>. In case of EDG3, additional exogenously added G<sub>q</sub> did not further improve the signal intensity. In case of EDG8, Yamazaki Y. et al. (2000) reported an S1P-induced mobilization of intracellular Ca<sup>2+</sup> but we failed to detect a significant Ca<sup>2+</sup> increase above basal levels in the absence of any cotransfected G-protein  $\alpha$  subunit. The reason for this discrepancy could be the cellular background, as Yamazaki Y. et al. (2000) reported that the Ca<sup>2+</sup> signal can be completely abolished in the presence of pertussis toxin (PTX), indicating the involvement of G<sub>i</sub>-type G-proteins. In this case the Ca<sup>2+</sup> signal would be elicited by bg, released from activated G<sub>a1g</sub> heterotrimers. The G<sub>a1</sub>-induced Ca<sup>2+</sup> signals are known to be

much smaller in intensity as compared with the Ca<sup>2+</sup> signals induced by bona-fide G<sub>q</sub>-linked receptors (Kostenis et al., 1997a; Kostenis et al., 1997b). It may be that detection of such Ca<sup>2+</sup> concentrations is beyond the sensitivity of the FLIPR system. EDG8 did not release intracellular Ca<sup>2+</sup> when stimulated with S1P (Fig.2), but gained the ability to mobilize Ca<sup>2+</sup> upon cotransfection with G $\alpha$ 16, a G-protein  $\alpha$ -subunit, known to couple GPCRs from different functional classes to the G<sub>q</sub>-PLC $\beta$  pathway or G $\alpha$ q15, a mutant G-protein  $\alpha$  subunit that confers onto G<sub>i</sub>-liked receptors the ability to stimulate G<sub>q</sub>. These results show that EDG8 as opposed to EDG 3 and 5 is not a bona-fide G<sub>q</sub>-coupled receptor but a functional cellular receptor for S1P. To check, whether the EDG8 receptor also reacts to related lysophospholipid mediators, we examined the abilities of lysophosphatidic acid (LPA), dihydroceramide 1-phosphate (DHS1P), sphingosylphosphorylcholine (SPC) and lysophosphatidylcholine (LPC) to increase intracellular Ca<sup>2+</sup> in CHO cells transiently transfected with the EDG8 receptor and the G-protein  $\alpha$  subunits G16 and Gq15 (Fig.3). Besides S1P, which was the most potent activator of EDG8, LPA and DHS1P evoked [Ca<sup>2+</sup>]i increases in concentrations of 100 and 1000 nM. SPC and LPC, respectively, failed to generate any significant response in concentrations up to 1  $\mu$ M.. These data show that EDG8 is a S1P preferring receptor, but also responds to related phospholipids like DHS1P or LPA, as has also been reported for EDG1, which is a high affinity receptor for S1P and a low affinity receptor for LPA .

26-04-2000

EP00108858.2

SPEC

Next, the expression pattern of the human EDG8 gene in human tissues was investigated by Northern blot analysis (Fig.4). Northern blot analysis shows EDG8 expression in several peripheral tissues. Tissues positive for EDG8 RNA were skeletal muscle, heart and kidney, lower abundance of RNA was seen in liver and placenta, no signal was obtained in brain, thymus, spleen, lung and peripheral blood leukocytes. In all tissues a single RNA transcript of 5.5 kb was observed after hybridization with a DIG-labelled EDG8 antisense RNA probe. EDG8 exhibits highest similarity to the rat nrg1-GPCR with an amino acid identity of 86.8% (Fig.1B) suggesting that it may be the human homolog of the rat nrg1 protein. However, the expression pattern of human EDG8 is quite different from the rat nrg1-receptor, which is found almost exclusively in brain. This finding suggests that EDG8 may represent a closely related but entirely different receptor from nrg1, rather than the human homolog. Never the less, it does not rule out the possibility that EDG8 and nrg1 are homologs with entirely different, species-dependent expression patterns.

As the first member of the EDG-family of GPCRs was cloned as an immediate early gene induced during the morphogenetic differentiation phase of angiogenesis (Hla and Maciag, 1990) and subsequently cloned from a human umbilical vein endothelial cell library exposed to fluid shear stress as an upregulated gene it is reasonable to assume that EDG receptors play an important role in the regulation of endothelial function. Therefore, the presence of EDG8 transcripts in several human endothelial cell lines was analyzed. RT-PCR analysis of human umbilical vein endothelial cells (HUVECs), human coronary artery endothelial cells (HCAECs), human microvascular endothelial cells of the lung (HMVEC-L) and human pulmonary artery endothelial cells (HPAEC) revealed EDG8 expression in all cell lines tested (Fig.5A). In Fig.5B it is shown that EDG8 specific primers indeed solely amplify EDG8 sequences and none of the related EDG1-7 sequences. These findings suggest that the presence of EDG8 in different peripheral organs may be due to its localization in endothelial cells; it does not rule out, however, that EDG8 transcripts occur in cell types other than endothelial cells.

The expression of EDG8 in addition to EDG1, 3, and 5 (Rizza et al., 1999) in HUVECS and several other endothelial cell lines is intriguing in view of all the reports

regarding S1P effects on endothelial cell signalling. Hisano et al. (1999) reported that S1P protects HUVECS from apoptosis induced by withdrawal of growth factors and stimulates HUVEC DNA synthesis; the authors derived a model for cell-cell interactions between endothelial cells and platelets but the S1P-receptor responsible for HUVEC-protection of apoptosis could not be identified. Rizza et al., 1999 reported that S1P plays a role in endothelial cell leukocyte interaction in that S1P induces expression of cell adhesion molecules in human aortic endothelial cells, allowing monocytes and neutrophils to attach. These effects were blocked by pertussis toxin, suggesting the involvement of a Gi-coupled S1P receptor. The responsible S1P-receptor subtype, however, could not be identified and the EDG8 receptor was not included at the time of this study. Expression profiling of all EDG receptors in individual cell lines and the use of EDG receptor subtype selective compounds will clearly be necessary to help determine the role of the individual S1P receptors in endothelial cell signalling mechanisms.

Finally, the mapping of EDG receptors in genomic sequences allowed to allocate a map position for four genes of this family (Tab.1). Interestingly, so far, four EDG-receptors including EDG8 are located on chromosome 19.

In conclusion, we isolated a new member of the EDG-family of G-protein coupled receptors, EDG8, and showed that it functions as a cellular receptor for sphingosine 1-phosphate. EDG8 could exclusively be detected in peripheral tissues like skeletal muscle, heart and kidney and several human endothelial cell lines. It is conceivable that the expression in endothelial cells may account for the broad tissue distribution of this receptor. The existence of at least eight EDG-receptors for lysophospholipids suggests that receptor subtype selective agonists and antagonists will essentially be necessary for a better understanding of the biology of lysophospholipids and their respective receptors.

#### Figure legends

Fig.1A: The nucleotide and deduced amino acid sequence of human EDG8. The deduced amino acid sequence is shown below the nucleotide sequence with the

10

nucleotide positions indicated on the left. The putative seven transmembrane domains are underlined.

Fig. 1B: Phylogenetic tree of the EDG-family of receptors. The phylogenetic tree depicted was derived by the neighbor joining method performed with the GCG program.

Fig. 1C: Alignment of the amino acid sequence of human EDG8 with the other EDG-family members. The amino acid sequence of EDG8 is compared with the EDG1-7 polypeptides (EDG1: accession number M 31210, EDG2: accession number U 80811, EDG3: accession number X 83864, EDG4: accession number AF 011486, EDG5: accession number AF 034780, EDG7: accession number AF 127138). The approximate boundaries of the seven putative transmembrane domains are boxed. Gaps are introduced to optimize the alignment.

Fig. 2: Mobilization of intracellular  $\text{Ca}^{2+}$  by S1P mediated by the EDG1, 3, 5 and 8 receptor in CHO cells, cotransfected with empty vector DNA as a control or the indicated G-protein  $\alpha$  subunits. Agonist-mediated changes of intracellular  $\text{Ca}^{2+}$  were measured with the FLIPR using the  $\text{Ca}^{2+}$ -sensitive dye FLUO4. Fluorescence of transfected cells loaded with FLUO4 was recorded before and after addition of S1P, applied in the indicated concentrations. Data are expressed as means of quadruplicate determinations in a single experiment. An additional experiment gave similar results.

Fig. 3: Effects of S1P, LPA and related lysophospholipid mediators on EDG8-mediated increase in intracellular  $\text{Ca}^{2+}$  measured with the FLIPR as described in the legend of Fig. 4. The different lipids were applied in concentrations of 10, 100 and 1000 nM, respectively. Data are means of quadruplicate determinations of a representative experiment. Two additional experiments gave similar results.

Fig. 4: Northern blot analysis of EDG8 in human tissues. Poly(A)+ RNA (1 $\mu$ g) from various human tissues (human multiple tissue Northern blots, CLONTECH) was hybridized with probes specific to human EDG8 (upper panel) and glyceraldehyde-3-

26-04-2000

EP00108858.2

SPEC

phosphate dehydrogenase, GAPDH, (lower panel) on a nylon membrane. The origin of each RNA is indicated at the top, the molecular mass of standard markers in kilobases (kb) is shown on the left.

**Fig.5A:** Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of EDG8 in different human endothelial cell lines (HUVECS: human umbilical vein endothelial cells; HCAEC: human coronary artery endothelial cells; HMVEC-L: human microvascular endothelial cells from lung; HPAEC: human pulmonary artery endothelial cells). EDG8-specific transcripts were detected in all endothelial cell lines. Agarose gel electrophoresis of the PCR products after 35 cycles of amplification is shown. Amplification with EDG8-specific primers yields a 522 bp EDG8-fragment as indicated by the arrow.

**Fig.5B:** PCR analysis of EDG8 primers for specificity of amplification of EDG8 sequences. Primers, specific for the EDG8 sequence, were checked for potential amplification of the related EDG1-7 sequences. The EDG8 specific 522 bp band occurred only when EDG8 was used as a template.

Table 1: Chromosomal localization of EDG-receptors 1-8.

Mapping of EDG receptors to genomic sequences allowed to derive a chromosomal assignment for EDG4, 5, 6 and 8. The chromosomal localization of EDG1-3 was obtained from the genecards datacollection,

EDG	Chromosomal localisation	according BAC AccNr.:
EDG1	1p21.1-21.3	AL161741
EDG2	9q31.1-32/ /18p11.3	AL157881/ /AP000882
EDG3	9q22.1-q22.2	
EDG4	19p12	NT_000939
EDG5	19	AC011611
EDG6	19p13.3	AC011547
EDG7	1p22.3-31.2	AL139822
EDG8	19	AC011481

## Examples

### Example 1: Molecular cloning of the human EDG8 receptor.

As the putative human EDG8 sequence is intronless, we cloned the receptor from human genomic DNA (CLONTECH, Palo Alto, CA, 94303-4230) via polymerase chain reaction (PCR). PCR conditions, established to amplify the EDG8 sequence were 94°C, 1 min followed by 35 cycles of 94°C, 30sec, 58C, 3 min, using GC-Melt Kit (CLONTECH, Palo Alto, CA). Primers designed to amplify the EDG8 sequence contained a HindIII site in the forward, and a EcoRI site in the reverse primer, respectively. The 1197 bp PCR product was cloned into the pCDNA3.1(+) mammalian expression vector (Invitrogen, Carlsbad, California) and sequenced in both directions.

### Example 2: Cell culture and Transfection.

CHO-K1 cells were grown in basal ISCOVE medium supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO<sub>2</sub> incubator. For transfections, 2 x 10<sup>5</sup> cells were seeded into 35-mm dishes. About 24 hr later cells were transiently transfected at 50-80% confluence with the indicated receptor and G-protein constructs (1μg of plasmid DNA each) using the Lipofectamine transfection reagent and the supplied protocol (GIBCO). 18-24 hr after transfection cells were seeded into 96well plates at a density of 50.000 cells per well and cultured for 18-24 additional hr until used in the functional FLIPR assays.

The cDNA for Gα16 was cloned from TF1 cells by RT-PCR and ligated into the pCDNA1.1 mammalian expression vector (Invitrogen). Murine wild type Gαq was cloned from cells by RT-PCR and inserted into the *Bam*H-I-*Nsi*I-sites of pCDNA1.1. To create the C-terminally modified Gα<sub>qi6</sub> subunit, in which the last five aa of wt Gαq were replaced with the corresponding Gα<sub>i</sub> sequence, a 175-bp *Bgl*II-*Nsi*I fragment was replaced, in a two piece ligation, with a synthetic DNA fragment, containing the desired codon changes. The correctness of all PCR-derived sequences was verified by sequencing in both directions.

**Example 3: Fluorometric Imaging Plate Reader (FLIPR) Assay.**

Twenty-four hours after transfection, cells were splitted into 96-well, black-wall microplates (Corning) at a density of 50,000 cells per well. 18-24 hr later, cells were loaded with 95 $\mu$ l of HBSS containing 20 mM Hepes, 2.5 mM probenecid, 4  $\mu$ M fluorescent calcium indicator dye Fluo4 (Molecular Probes) and 1% fetal bovine serum for 1 h(37°C, 5% CO<sub>2</sub>). Cells were washed three times with HBSS containing 20 mM Hepes and 2.5 mM probenecid in a cell washer. After the final wash, the solution was aspirated to a residual volume of 100  $\mu$ l per 96 well. Lipid ligands were dissolved in DMSO as 2 mM stock solutions (treated with ultrasound when necessary) and diluted at least 1:100 into HBSS containing 20 mM HEPES, 2.5 mM probenecid and 0.4 mg/ml fatty acid free bovine serum albumine. Lipids were aliquoted as 2X solutions into a 96 well plate prior to the assay. The fluorometric imaging plate reader (FLIPR, Molecular Devices) was programmed to transfer 100  $\mu$ l from each well of the ligand microplate to each well of the cellplate and to record fluorescence during 3 min in 1 second intervals during the first minute and 3 second intervals during the last two minutes. Total fluorescence counts from the 18-s to 37-s time points are used to determine agonist activity. The instrument software normalizes the fluorescent reading to give equivalent initial readings at time zero.

**Example 4: Northern Blot analysis.**

Human multiple tissue Northern blots were purchased from CLONTECH (Palo Alto, CA, 94303-4230, USA) antisense RNA probes were generated by subcloning nucleotides 279-1197 of the coding region into the Bam HI-Eco RI sites of the expression vector PSPT18 (Roche Diagnostics, Mannheim, Germany) and subsequent random priming with a DIG-RNA Labeling kit (Roche Diagnostics, Mannheim, Germany), using T7 RNA polymerase. Hybridization was carried out at 68°C for 16 h in hybridization buffer (Dig Easy Hyb Roche Diagnostics, Mannheim, Germany). Each blot was washed , blocked and detected as indicated in the standard protocol with the DIG Wash and Block Buffer set (Roche Diagnostics, Mannheim, Germany) and treated with 1 ml CSPD ready-to-use(Roche Diagnostics, Mannheim, Germany) for 15 min , 37°C and developed for 5 min on the Lumiimager (Roche). Finally, each blot was stripped (50 % formamid,5% SDS, 50 mM Tris/HCl

pH 7,5 ; 80° C, 2x 1 hour) and rehybridized with a GAPDH antisense RNA probe as an internal standard.

#### Example 5: RNA Extraction and RT-PCR.

RNA was prepared from different endothelial cell lines (HUVECS, HCAEC, HMVEC-L, HPAEC) using the TRizol reagent (Hersteller, Lok.). Briefly, for each endothelial cell line, cells of a subconfluent 25 cm<sup>2</sup> tissue culture flask were collected in 2,5ml TRizol and total RNAs were extracted according to the supplied protocol. The purity of the RNA preparation was checked by verifying the absence of genomic DNA. An aliquot of RNA, corresponding to ~5µg, was used for the cDNA generation using MMLV reverse transcriptase and the RT-PCR kit from STRATAGENE. RT-PCR was carried out in a volume of 50 µl, the RT-PCR conditions were set to 65°C for 5 min, 15min at RT, 1 hour at 37°C, 5 min at 90°C, chill on ice.

The cDNA templates for the PCR reactions (35 cycles of 94°C for 30 sec, 68°C for 3 min) were the reverse transcribed products of RNAs isolated from human endothelial cell lines (HUVECS, HCAEC, HMVEC-L, HPAEC). Typically, 1-5 µl of reverse transcribed cDNAs were used as templates for the PCR reactions.

#### Example 6: Sources of materials.

1-oleoyl-LPA, sphingosin 1-phosphate (S1P), dihydroosphingosin 1-phosphate (DHS1P), lysophosphatidylcholine (LPC), sphingosylphosphorylcholine (SPC) and fatty acid free BSA were from SIGMA (P.O.Box 14508, St. Louis, Missouri 63178). CHO-K1 cells were obtained from the American Type culture collection (ATCC, Manassas, Virginia), cell culture media and sera from GIBCO BRL (Gaithersburg, MD), the Ca fluorescent dye FLUO4 and pluronic acid from Molecular devices (Sunnyvale CA 94089-1136, USA) human northern blot membrane from CLONTECH (1020 East Meadow Circle, Palo Alto, California 94303-4230, USA.), commercially available cDNAs (heart, fetal heart, left atrium, left ventricle, kidney, brain, liver, lung, aorta) from Invitrogen, oligonucleotides from MWG-Biotech AG (Ebersberg, Germany), the RT-PCR kit from SIGMA, the GC-melt PCR kit from Clontech (Palo Alto, CA), the expression plasmid pcDNA3.1 for EDG8 and pCDNA1.1 for expression of G-protein α subunits from Invitrogen (Carlsbad, CA 92008), competent DH5α from GIBCO and MC 1063 from Invitrogen.

**References**

- Durieux ME, and Lynch KR (1994); Trends Pharmacol. Sci. 14: 249-254  
Gohla A et al. (1999). J. Biol. Chem. 274: 17901-17907  
Jalink K et al. (1994) Biochim. Biophys. Acta 1198: 185-196  
Moolenaar WH (1994) Trends Cell Biol. 4: 249-254  
Moolenaar WH et al. (1997) Current opinion in cell biology 9: 168-173  
Morris AJ (1999) Trends Pharmacol. Sci. 20:393-395

**List of non-standard abbreviations:**

S1P, sphingosine 1-phosphate; LPA, lysophosphatidic acid; dHS1P, dihydro sphingosine 1-phosphate; SPC, sphingosylphosphorylcholine; LPC, lysophosphatidylcholine; GPCR, G-protein-coupled receptor; G-protein, guanine nucleotide-binding protein; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular Calcium concentration, RT-PCR, reverse transcription polymerase chain reaction; bp, base pair; ORF, open reading frame; EST, expressed sequence tag; FAF-BSA, fatty acid free bovine serum albumine; HUVECS, Human umbilical vein endothelial cells; HCAEC, human coronary artery endothelial cells; HMVEC-L, human microvascular endothelial cells from lung; HPAEC, human pulmonary artery endothelial cells.

**Table 2:****SEQ ID NO. 1: Nucleotide sequence of human EDG8**

1 ATGGAGTCGGGCCTGCTGGGCCGGCGCCGGTGAGCGAGGTACATCGTCCTGCATTACAAC  
61 TACACCGGCAAGCTCCGGGTGCGCGCTACCAGCCGGTGCCGGCCTGCGCGCCGACGCC  
121 GTGGTGTGCCCTGGCGGTGTGCGCCTCATCGTGTAGAGAAATCTAGCCGTGTTGGTG  
181 CTCGGACGCCACCCCGCGCTTCCACGCTCCCATGTTCCCTGCTCCTGGGCAGCCTCACGTTG  
241 TCGGATCTGCTGGCAGGCGCCCTACGCCGCCAACATCCTACTGTCGGGGCCGCTCACG  
301 CTGAAACTGTCCCCCGCGCTCTGGTTCGCACGGGAGGGAGGCGTCTCGTGGCACTCACT  
361 GCGTCCTGCTGAGCCTCTGGCCATCGCGCTGGAGCGCAGCCTCACCATGGCGCGAGG  
421 GGGCCCGCGCCCGTCTCCAGTCGGGGCGCACGCTGGCGATGGCAGCCGCGGCTGGGC  
481 GTGTCCTGCTCCCTGGGCTCTGCCAGCGCTGGCTGGAATTGCCCTGGTCGCCCTGGAC  
541 GCTTGCTCCACTGTCTTGCCGCTCTACGCCAAGGCCTACGTGCTCTCGCTGCTGCC

601 TTCTGTGGGCATCCTGGCCGCTATCTGTGCACTCTACCGCGCGCATCTACTGCCAGGTACGC  
661 GCCAACGCGCGGCGCCTGCCGGCACGGCCCAGGACTGCCGGGACCACCTCGACCCGGCG  
721 CGTCGCAAGCCGCGCTCGCTGGCCTTGCTGCGCACGGCTCAGCGTGGTGCCTGGCCTT  
781 GTGGCATGTTGGGGCCCCCTTCTGCTGCTGTTGCTGACGTGGCGTCCCCGGCGCG  
841 ACCTGTCCGTACTCCTGCAGGCCGATCCCTCCTGGACTGCCATGCCAACTCACTT  
901 CTGAACCCCATCATCACACGCTCACCAACCGCGACCTGCGCACGCGCTCCTGCGCTG  
961 GTCTGCTGCGGACGCCACTCCTGCAGAGACCCGAGTGGCTCCCAGCAGTCGGCGAGC  
1021 GCGGCTGAGGCTTCCGGGGGCTGCSCGCTGCCTGCCCCCGGGCTTGATGGAGCTTC  
1081 AGCGGOTCGGAGCGCTCATCGCOCCAGCGCACGGCTGGACACCAGCGGCTCCACAGGC  
1141 AGCCCCGGTGCACCCACAGCCGCCGGACTCTGGTATCAGAACCGGCTGCAGACTGA

Table 3:

SEQ ID NO. 2: Amino acid sequence of human EDG8

M	E	S	G	L	L	R	P	A	P	V	S	E	V	I	V	L	H	Y	N
Y	T	G	K	L	R	G	A	R	Y	Q	P	G	A	G	L	R	A	D	A
V	V	C	L	A	V	C	A	F	I	V	L	E	N	L	A	V	L	L	V
L	G	R	H	P	R	F	H	A	P	M	F	L	L	L	G	S	L	T	L
S	D	L	L	A	G	A	Y	A	A	N	I	L	L	S	G	P	L	T	
L	K	L	S	P	A	L	W	F	A	R	E	G	G	V	F	V	A	L	T
A	S	V	L	S	L	L	A	I	A	L	E	R	S	L	T	M	A	R	R
G	P	A	P	V	S	S	R	G	R	T	L	A	M	A	A	A	W	G	
V	S	L	L	L	G	L	L	P	A	L	G	W	N	C	L	G	R	L	D
A	C	S	T	V	L	P	L	Y	A	K	A	Y	V	L	F	C	V	L	A
F	V	G	I	L	A	A	I	C	A	L	Y	A	R	I	Y	C	Q	V	R
A	N	A	R	R	L	P	A	R	P	G	T	A	G	T	T	S	T	R	A
R	R	K	P	R	S	L	A	L	L	R	T	L	S	V	V	L	L	A	F
V	A	C	W	G	P	L	F	L	L	L	L	D	V	A	C	P	A	R	
T	C	P	V	L	L	Q	A	D	P	F	L	G	L	A	M	A	N	S	L

17

L N P I I Y T L T N R D L R H A L L R L  
V C C G R H S C G R D P S G S Q Q S A S  
A A E A S G G L R R C L P P G L D G S F  
S G S E R S S P Q R D G L D T S G S T G  
S P G A P T A A R T L V S E P A A D \*

26-04-2000

EP00108858.2

SPEC

**Claims:**

1. An isolated polynucleotide comprising a nucleotide sequence that has at least 80 % identity to a nucleotide sequence encoding the polypeptide of SEQ ID NO. 2 or the corresponding fragment thereof; or a nucleotide sequence complementary to said nucleotide sequence.
2. The polynucleotide of claim 1 which is DNA or RNA.
3. The polynucleotide of claim 1 wherein said nucleotide sequence is at least 80 % identical to that contained in SEQ ID NO. 1.
4. The polynucleotide of claim 3 wherein said nucleotide sequence is contained in SEQ ID NO. 1.
5. The polynucleotide with sequence SEQ ID NO. 1.
6. The polynucleotide of claim 1 wherein said encoding nucleotide sequence encodes the polypeptide of SEQ ID NO. 2 or a fragment thereof.
7. EDG8 DNA or RNA molecule comprising an expression system wherein said expression system is capable of producing a polypeptide or a fragment thereof having at least 80 % identity with a nucleotide sequence encoding the polypeptide of SEQ ID NO. 2 or said fragment when said expression system is present in a compatible host cell.
8. A host cell comprising the expression system of claim 7.
9. A process for producing a EDG8 polypeptide or fragment comprising culturing a host claim 8 and under conditions sufficient for the production of said polypeptide or fragment.

10. The process of claim 9 wherein said polypeptide or fragment is expressed at the surface of said cell.
11. Cells produced by the process of claim 10.
12. The process of claim 9 which further includes recovering the polypeptide or fragment from the culture.
13. A process for producing a cell which produces a EDG8 polypeptide or a fragment thereof comprising transforming or transfecting a host cell with the expression system of claim 7 such that the host cell, under appropriate culture conditions, produces a EDG8 polypeptide or fragment.
14. EDG8 polypeptide or a fragment thereof comprising an amino acid sequence which is at least 80 % identical to the amino acid sequence contained in SEQ ID NO. 2.
15. Polypeptide of claim 14 which comprises the amino acid sequence of SEQ ID NO. 2, or a fragment thereof.
16. EDG8 Polypeptide or fragment prepared by the method of claim 12.
17. A process for diagnosing a disease or a susceptibility to a disease related to expression or activity of EDG8 polypeptide comprising:
  - c) determining the presence or absence of mutation in the nucleotide sequence encoding said EDG8 polypeptide in the genome of said subject; and/or
  - d) analyzing for the presence or amount of the EDG8 polypeptide expression in a sample derived from said subject.
18. A method for identifying compounds which bind to EDG8 polypeptide comprising:
  - c) contacting a cell as claimed in claim 11 or a part thereof with a candidate compound; and
  - d) assessing the ability of said candidate compound to bind to said cells.

19. The method of claim 18 which further includes determining whether the candidate compound effects a signal generated by activation of the EDG8 polypeptide at the surface of the cell, wherein a candidate compound which effects production of said signal is identified as an agonist.
20. The method of claim 18 which further includes determining whether the candidate compound effects a signal generated by activation of the EDG8 polypeptide at the surface of the cell, wherein a candidate compound which effects production of said signal is identified as an antagonist.
21. An agonist identified by the method of claim 19.
22. An antagonist identified by the method of claim 20.
23. The method of claim 18 which further includes contacting said cell with a known agonist for said EDG8 polypeptide; and determining whether the signal generated by said agonist is diminished in the presence of said candidate compound, wherein a candidate compound which effects a diminution in said signal is identified as an antagonist for said EDG8 polypeptide.
24. A method as claimed in claim 23, wherein the known agonist is S1p, LPA and/or DHS1P.
25. An antagonist identified by the method of claim 23 or 24.
26. Method of preparing a pharmaceutical composition comprising
  - a) identifying a compound which is an agonist or an antagonist of EDG8,
  - b) preparing the compound, and
  - c) optionally mixing the compound with suitable additives.
27. Pharmaceutical compound prepared by a process of claim 26.

## FIG 1A:

1 ATGGAGTCGGGGCTGCTGCCGCCGCCGCCGGTGGCGAGGTATCGTCCTGCATTACAAC  
M E S G L L R P A P V S E V I V L H Y N

61 TACACCGGAAGCTCCGGTGGCGCTACCAGCCGGTGCCGGCCTGCCGCCGACGCC  
Y T G K L R G A R Y Q P G A G L R A D A

121 GTGGTGTGCCCTGGCGGTGTGCCCTCATCGCTAGAGAATCTAGCCGTGTTGGTG  
V V C L A V C A F I V L E N L A V L L V

181 CTCGGACGCCACCCCGCGCTTCCACGCTCCATGTTCTGCTCTGGGAGCCTCACGTTG  
L G R H P R F H A P M F L L L G S L T L

241 TCGGATCTGCTGGCAGGCCGCCCTACGCCGCCAACATCCTACTGTCGGGCCGCTCACG  
S D L L A G A A Y A A N I L L S G P L T

301 CTGAAACTGTCCCCCGCGCTCTGGTCGACGGAGGGAGGGCTTCGTGGCACTCACT  
L K L S P A L W F A R E G G V F V A L T

361 GCGTCCGTGCTGAGCCTCCATGGCCTCGCGCTGGAGCGCAGCCTCACCATGGCGCGAGG  
A S V L S L L A I A L E R S L T M A R R

421 GGGCCCGCGCCCGTCCAGTCGGGGCGCACGCTGGCGATGGCAGCCGGCCTGGGC  
G P A P V S S R G R T L A M A A A A W G

481 GTGTCGCTGCTCCCTCGGGCTCTGCCAGCGCTGGCTGGAATTGCCCTGGGTGCCCTGGAC  
V S L L G L P A L G W N C L G R L D

541 GCTTGCTCCACTGTCTTGGCGCTCTACGCCAAGGCCAACGTGCTCTTCGTGCTCGCC  
A C S T V L P L Y A K A Y V L F C V L A

601 TTCTGGGCATCTGGCGCTATCTGTGCACTCTACGCCGCTACTGCCAGGTACGC  
F V G I L A A I C A L Y A R I Y C Q V R

661 GCCAACGCCGGCGCCCTGCCGGCACGGCCGGACTGCCGGACCACCTGACCCGGCG  
A N A R R L P A R P G T A G T T S T R A

721 CGTCGCAAGCCGCGCTCGCTGGCCTTGTGCGCACGCTCAGCGTGGTGCCTGGCCTT  
R R K P R S L A L L R T L S V V L L A F

781 GTGGCATGTTGGGGCCCCCTTCCCTGCTGCTGTTGCTGACGTGGCGTCCCCGGCGC  
V A C W G P L F L L L D V A C P A R

841 ACCTGCTCTGTACTCTGCAGGCCGATCCCTGGACTGCCATGCCAACACT  
T C P V L L Q A D P F L G L A M A N S L

901 CTGAACCCCCTACATCACGCCACCAACGCCACCTGCCACGCCGCTCTGCCCTG  
L N P I I Y T L T N R D L R H A L L R L

961 GTCTGCTGCCGACGCCACTCTGCCAGAGACCCGAGTGGCTCCAGCAGTCGGCGAGC  
V C C G R H S C G R D P S G S Q Q S A S

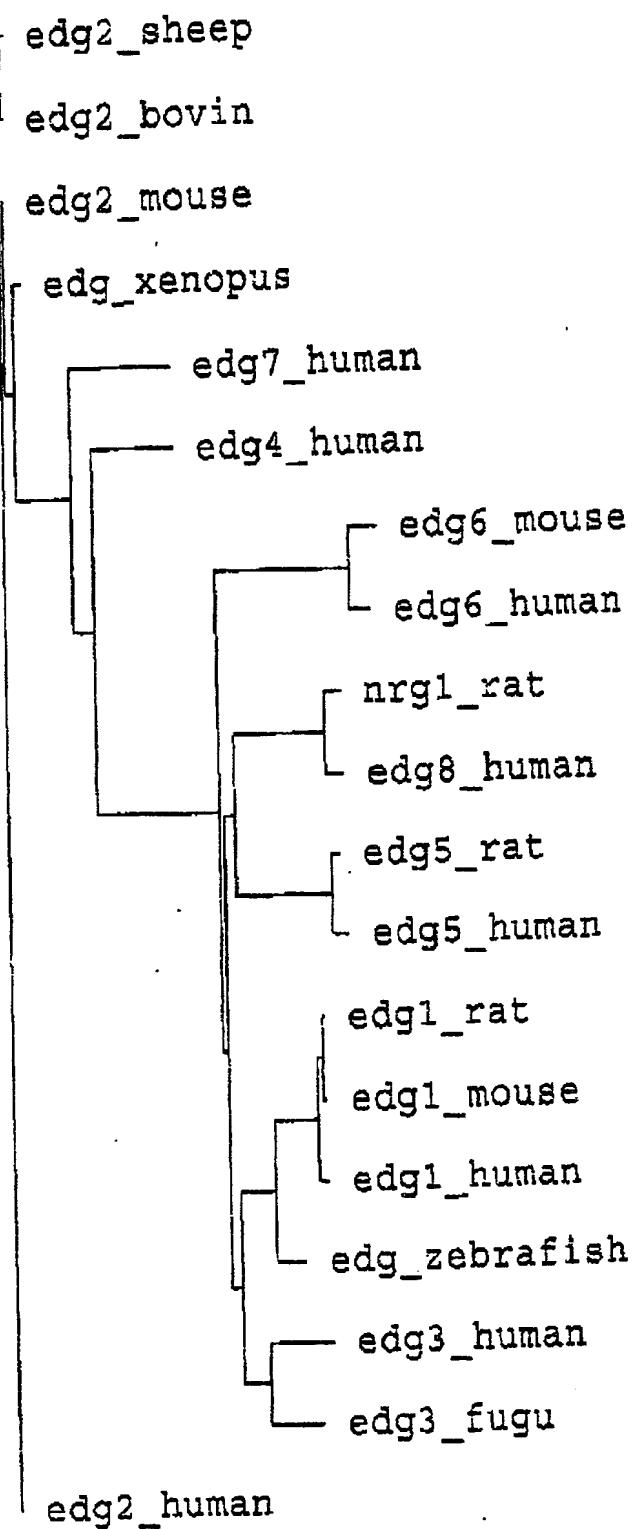
1021 GCGGCTGAGGCTTCCGGGGCTGCCCGCTGCCCTGCCCTGGGAGCTTC  
A A E A S G G L R R C L P P G L D G S F

1081 AGCGGCTCGGAGCGCTCATCGCCCCAGCGCGACGGCTGGACACCAGCGGCTCCACAGGC  
S G S E R S S P Q R D G L D T S G S T G

1141 AGCCCCGGTGCACCCACAGCCGCCGGACTCTGGTATCAGAACGGCTGAGACTGA  
S P G A P T A A R T L V S E P A A D \*

22

Fig. 1B



23

FIG 1C:

1 60  
 edg2\_human MAAISTSTPV ISQPOFTAMM EPQCFYNESI AFFYNRSGKH LAT.EKNTVS KLVMLG..GI  
 edg7\_human ~~~~~ MN E..CHYDHXM DFFYNRSNTP TVD.DW.TGT KLVIVLCVGT  
 edg4\_human ~~~~~ MVI MGQQCYNETI GFYFNNSGKE LSS.HWR..P RDVVVVVALGL  
 edg1\_human ~~~~MGPTS VPLVKAHRSS VSDYVNYDII VRHYNYTGKL ..NISADKEN SIKLTSVVFI  
 edg3\_human ~~~~MATALPPR LQPVARGNETL REHYCYVGKL AGRLEKEASEG S.TLTTCVLF  
 edg5\_human ~~~~MGSL YSEYLNPKV QEHYNYTKE ..TLETQETT SRQVABAPIV  
 edg8\_human ~~~~MESGL LRPAPVSEVI VLHYNYTGKL RG.ARYQPQA GLRADAVVCL  
 edg6\_human ~~~~MNATG TPVAPESCQQ LAAGGHSLI VLHYNHSGRL AGR.GGPEDG GLGALRGISV

61 120  
 edg2\_human TVCIFIMLAN LLVMVIAIYVN RRFHFPPIYL MANLAAADFF AGIAYFYL MF NTGPNTRRLT  
 edg7\_human SLVIAAVIKN RKFHFPPIYL LAMLAADFF AGIAYFYL MF NTGPVSKLT  
 edg4\_human TVSVLVLITLN LLVIAIAASN RRFHFPPIYL LGNLAADFL AGVAYFL MF HTGPRTARLS  
 edg1\_human LIICCFILEN IFVLLTIWKT KKPFHREMYFY IGNJALS DLL AGVAYTANLL LSGATTYKLT  
 edg3\_human VICSFIVLEN LMVLIAITWN NKFHNRMYFF IGNJALCDLL AGIAYKVNL MSGKKTFSLS  
 edg5\_human ILCCAIVVEN LLVILIAVARN SKFHSAMYLF LGNLAAS DLL AGVAFVANTL LSGSVTLRLT  
 edg8\_human AVCAFIVLEN LAVLVLIGRH PRFMAEMFLL LGSLTLS DLL AGAAYAANIL LSGPLTLKLS  
 edg6\_human AASCLVVEN LLVILAAITSH MRSRRMVYYC LVNITLSDLL TGAAYLANVL LSGARTFRLA

121 180  
 edg2\_human VSTWLRLRQL IDTSILTASVA NLLATAIERH ITVFR.MQLH TRMSNRRVVV VIVVIWMTAI  
 edg7\_human VNRWFLRLRQL LDSSILTASLT NLLVIAVERH MSIMR.MRVH SNLTKKRVTI LILLVWAI  
 edg4\_human LEGWNFLRLRQL LDTSILTASVA TLLAIAVERH RSWKA.VOLH SRLPRGRVVM LIVGVVVAAL  
 edg1\_human PAQWFRLREGS MFVAlSASFV SLLAIAIERY ITMLK.MKLH NGNNFRFLPL LISACWVSL  
 edg3\_human PTWWFLREGS MFVALGASTC SLLAIAIERH LTMIK.MRPY DANKRHRVFL LIGMCWLI  
 edg5\_human PVQWFAREGG ASITISASFV SLLAIAIERH VAIAK.VKLY GSDKSCRMIL LIGASWLI  
 edg8\_human PALWFAREGG VFVALTASBV SLLAIALERS LTMAR.RGPA DVSSRGRTLA MAAAANGVSL  
 edg6\_human PAQWFRLREGL IFTALAASFT SLLFTAGERF ATMVRPVAA SAKTTSRVYC FIGLCWLLAA

181 240  
 edg2\_human VMGAIPSVGW NCICDIENS NMAPLYSDSY LVFWAIFNLV TFVVMVVLYA HIFGYVRQRT  
 edg7\_human FNGAVPTLGNW NCICNISACS SLAPITYRSY LVFWTVSNLM AFLIMUVVYL RIYVYVURKRT  
 edg4\_human GLGLLPANHWS HCLCALDRCS RMAPLLRSY LAVWALESLL VFLMVAVYT RIFFYVRRTV  
 edg1\_human ILGGGLPIMGN NCISALSSCS TVLFLYHKKY ILFCTTVFTL LLLSIVILYC RIYSLVRTRS  
 edg3\_human TLGALPILGW NCLHNLPDCS TILFLYSSKKY IAFCISIFTA ILTIVILYA RIYFLVKSSS  
 edg5\_human VLGGLPILGW NCLGRHLAECS TVLFLYAKHY VLCVVTIFSI ILLAIVALYV RIYCVVRSSH  
 edg8\_human LLGLLPALGW NCLGRLDACS TVLFLYAKRAY VLCVLAFTVG ILAICALYA RIYCOVRA  
 edg6\_human LLGMLPILLW NCLCAFDRCS SLLFLYSKRY ILFCLVIFAG VLATIMGLYQ AIFRLVQASG

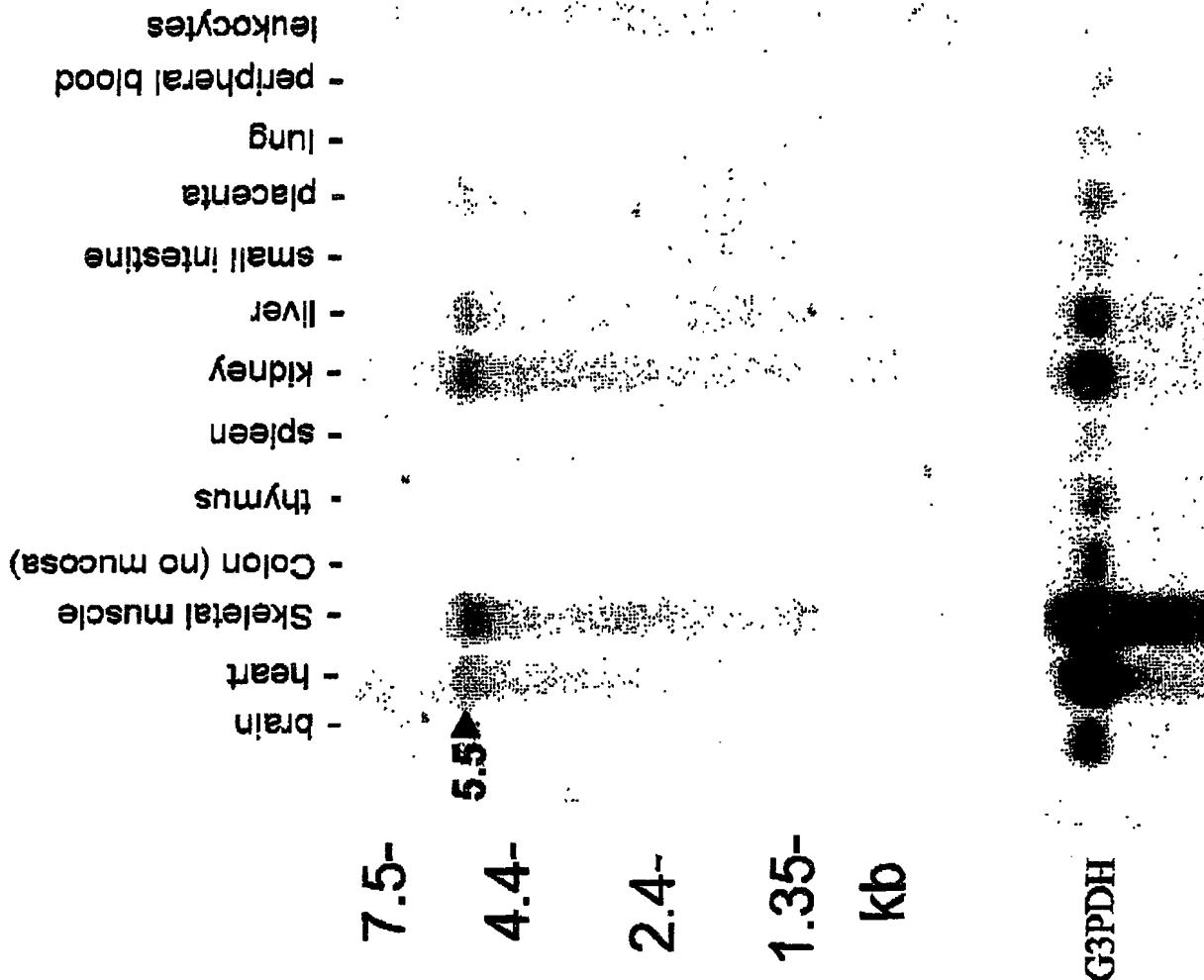
241 300  
 edg2\_human MRMSRHSSGP R..... RNR DTMSLKLKTV VIVLGAFIIC WTPGLVLLLL D.VCCP..QC  
 edg7\_human NVLSPRTSGB I..... SRR RTFMKLMKTV MTVLGAFVUC WTPGLVULL DGLNCR..QC  
 edg4\_human QRMAEHVSCH P..... RYR ETTLSLVKT VVILGAFVUC WTPGQVULL DGLGCE..SC  
 edg1\_human RRLTFR... KNISKASRS GENVALLRTV IIVLVSFIAC WAPLFILLLL DV.GCKVKTC  
 edg3\_human RKVANH... NN..... S ERSMALLRTV VIVVSVFIAC WSPLFILFL DV.ACIVQAC  
 edg5\_human ADMA..... A PTLALLRTV TIVLGVFTVC WLPAFSLLLL DV.ACIVHSC  
 edg8\_human RRLPARPGTA GTTSTRARRK PRSLALLRTL SVVLLAFVAC WGPLFILLLL DV.ACIPARTC  
 edg6\_human QKAP..... RPAARRK ARR..LLKTV LNILLAFLVC WGPLFGLLLA DVFGSNLWAQ

301 360  
 edg2\_human DVLAYEKFFL LLAEFNSAMM PIISYSDRKE MSATFRQILC CORSENPTGP TESSDRSASS  
 edg7\_human GVQEVKRMEL LLLALN9VVA PIISYSDDED MYGMKRMIC CFSQENP... ERRPSR  
 edg4\_human NYLAVERKYFL LLAEANSLVA AAVYSCRDAE MRRTFRRLIC CACLRQSTRE SVHYTSSAQG  
 edg1\_human DILFRAEYFL VLAVLNSGTM PIYTTLTNKE MRRAFIRIMS CCKCPGSD..  
 edg3\_human FILFLRAQWFI VLAVLNSAMM PIYTTLASKE MRRAFRFLV .CNC.IVR..  
 edg5\_human PIYLYAHYFF AVSTLNSLLN PIYTWTWSRD LRREVRPLQ CWRPGVGV..  
 edg8\_human PVLLQADPFL GLAMANSLLN PIYTTLNRD LRHALLRVCG CGRHSGCRDP SGS..QOSAS  
 edg6\_human EYLRGMDWIL ALAVLNSAVN PIYTFSRSRE VCRAVLSFLC CGCLRLGMRG PGDCLARAVE

361 418  
 edg2\_human LNHTILAGVH SNDHSVV---  
 edg7\_human IPSTVLSRSD TGSOYIEDSI SQGAVCNKST S-----  
 edg4\_human GASTRIMLPE NGHPLMTPPF SYLELQRYYA SNKSTAPUDL NVLLAQPNQQ D-----  
 edg1\_human AGKFKRPIIA GMFDSRSK.. SDNSSSHFKQ DEGDNPETIM SSGNVNNSSS-----  
 edg3\_human RGARASPIQP ALDPSRSKSS SBNNSSHSPK VKEDLPHDTP SSCIMDKNAA LQNGIFCN  
 edg5\_human GRRVGTGPH HLLPLRS888 LERGMNMPTS PTFLEGNTVV -----  
 edg8\_human AAEASGGLRR CLPPGLDG8F SGSERSSFPQ DGLDTSGSTG SPGAPTRART LVSEPAAD  
 edg6\_human AHSGAETTDS SLRP.RDSFR GSRSLSFRMR EPLSSISSVR SI-----

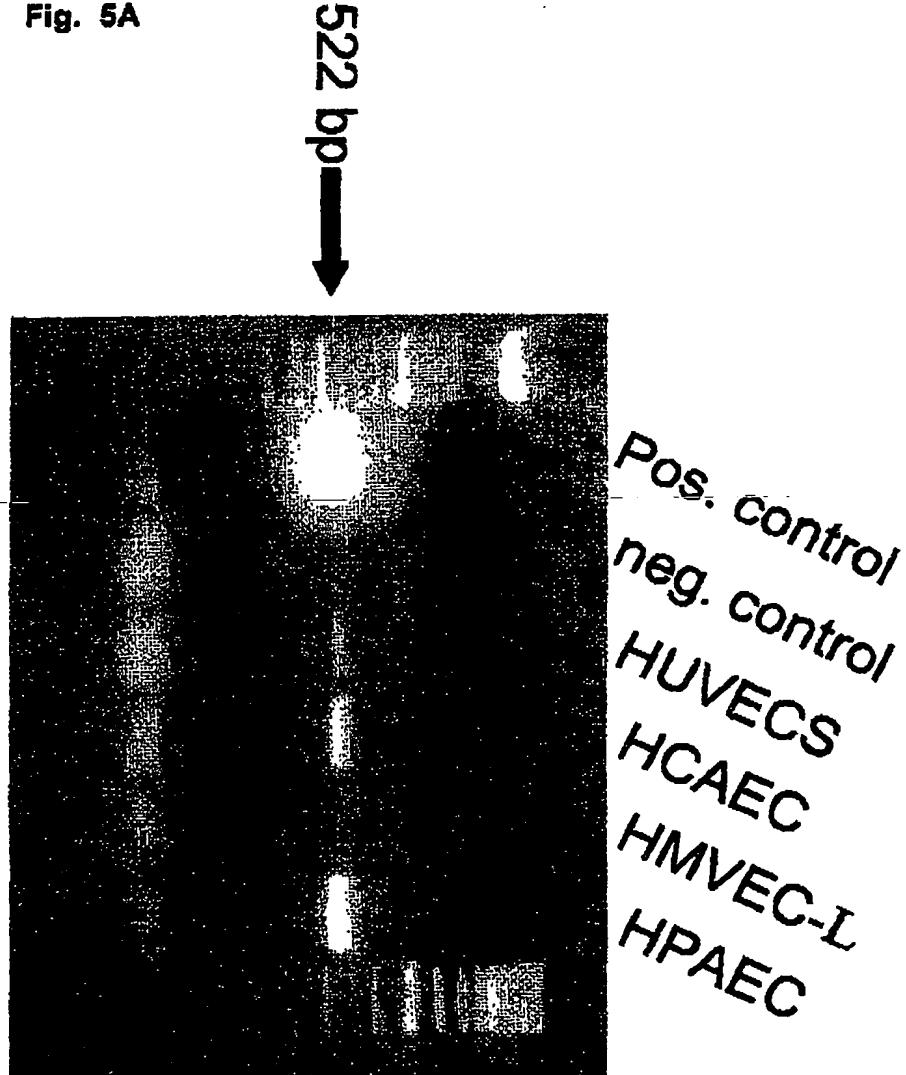
6/8

Fig. 4



7/8

Fig. 5A

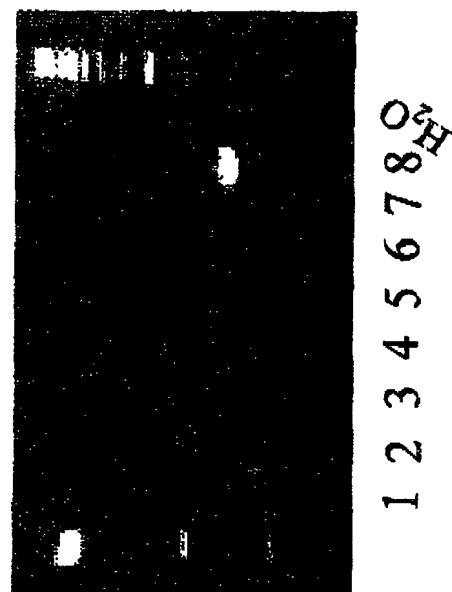


26-04-2000

EP00108858.2

SPEC

8/8

**Fig. 5B**

1 2 3 4 5 6 7 8 H<sub>2</sub>O